Attachments of # 3 D3 10/23/02

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 3/06, C12N 9/96

A1

(11) International Publication Number: WO 94/10191

(43) International Publication Date: 11 May 1994 (11.05.94)

(21) International Application Number: PCT/DK93/00344

(22) International Filing Date: 28 October 1993 (28.10.93)

(30) Priority data:
1328/92 30 October 1992 (30.10.92) DK

(71) Applicant *(for all designated States except US)*: NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : LOEVBORG, Uffe [DK/DK]; Grantofteparken 60, DK-2750 Ballerup (DK).

(74) Common Representative: NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).

(81) Designated States: BR, CA, FI, JP, KR, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: LOW ALLERGENIC PROTEINS

(57) Abstract

Through changes in size of protein(s) and monitoring and testing the effect hereof molecules are selected that has lowered potential for stimulating an allergic reaction in humans and animals. Methods for production either during the production of the protein(s) itself of after this in purification, processing or confectioning of the protein(s) are advised. The changed proteins can be for use in industry, household, food/feed or medicine.

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Title: LOW ALLERGENIC PROTEINS

FIELD OF THE INVENTION

This invention relates to low allergenic proteins and methods for changing proteins, especially industrially produced proteins including enzymes, physically or chemically to become less allergenic than their precursors. Furthermore the invention relates to compositions containing such proteins, and the use of the low allergenic proteins or the compositions in various fields including household, industry food/feed and medicine.

BACKGROUND OF THE INVENTION

An increasing number of proteins are being produced industrially, for use in industry, household, food/feed or medicine. Being proteins they are capable of stimulating the immune systems of animals including humans.

Depending on the way of presentation proteins can elicit the production of different kinds of antibodies and/or a cellular response. At least one of these routes can give adverse effects in animals including humans.

The production of IgE (in humans, and molecules with comparable effects in animals) can lead to an allergic state, giving symptoms like rhinitis, conjunctivitis or other.

Generally, most animals including humans are not exposed to these proteins to a degree that will generate adverse effects, but certain risk groups exist for which these phenomena are of significant importance.

The risk groups can be personnel in industrial production or

research departments etc.

The increased use of such protein products could, however, lead to persons using the proteins as consumers or otherwise being affected. Furthermore, the effect of these proteins after their use and disposal on the environment and the animals including humans living there is expected to increase.

With industrial proteins present methods to avoid problems in connection with allergic reactions, generally consist of various ways of immobilizing, granulating, coating or dissolving the proteins, to avoid especially protein in dust form from stimulating the immune system.

There will anyhow still be a risk of having protein dust or dissolved protein in aerosol form. Therefore some release of protein can occur leading to a possible allergic reaction in those sensitive to such exposure.

Another way of diminishing the problem has been to select proteins of human origin for production, f.ex. in bacteria. This may alleviate some problems for humans, but not for animals. Furthermore, it will in many cases not be possible to find proteins with the desired properties of human origin, wherefore other origin has to be considered. This can be either human proteins that are altered in one or more positions in the molecule, giving performance that is desired. Or it might be molecules from other species, including bacteria, mold etc. All the latter groups of products will have potency for immune stimulation.

A further proposition for decreasing allergenicity has been to reduce the size of the protein molecules (see e.g. JP Patent Publication No. 4112753, or Research Disclosure No. 335102). This is, however, a solution that is only available when the activity of the protein is without importance, or in such rare cases, where the activity of the protein in question is

retained in spite of a breakdown of the protein.

Recently the use of protein engineering has been suggested to reduce the allergenicity of proteins through epitope mapping and subsequent change of the allergenic epitopes (see International Patent Publication No. WO 92/10755). This procedure, however, usually requires a large investment in work and development.

In the medicinal field suggestions have been made of diminishing the antigenicity or allergenicity of proteins through the attachment of one or more polymer molecules to the protein. This usually has the effect of interfering with the interactions of the protein with other macromolecular structures.

Such a conjugate may also exhibit novel properties: e.g. EP Patent No. 38 154 discloses conjugates of allergens with polysarcosine which have immunosuppressive properties.

It has been found that the attachment of proteins to polymers in general has the effect of lowering the activity of the protein or interfering with the interaction between the protein and its substrate.

EP Patent No. 183 503 discloses a development of the above concept by providing conjugates comprising pharmaceutically useful proteins linked to at least one water-soluble polymer by means of a reversible linking group.

SUMMARY OF THE INVENTION

The primary object of the invention is to provide novel low allergenic proteins, especially low allergenic proteins comprising an oligomeric form of the parent monomeric protein which oligomer has substantially retained its activity.

The invention furthermore relates to methods for changing proteins, especially industrially produced proteins including enzymes, physically or chemically to become less allergenic than their precursors. The change is brought about through a change of the effective size of the protein to a size above the possible size for penetration of the membrane barrier of the wet epithelias, while substantially retaining the activity of the protein for its desired use.

According to the invention the change in size is effected through various methods that are more or less known *per se* in order to obtain oligomeric forms of the protein in question, whereafter the oligomeric proteins are tested for their desired activity. If this is satisfactory the proteins are subsequently tested for their allergenicity.

The invention in this aspect also relates to the selection of methods for increasing the size of proteins, without disabling the later use of these proteins. This is achieved either by leaving the activity/functionality intact during the binding/-linking of the protein(s), or through the protein in question resuming the original activity/functionality after a reversion to its monomeric form, e.g. by splitting off the binding or linking molecule(s).

According to one special aspect of the invention the increase in size is obtained through the insertion of a DNA sequence coding for a protein comprising a multiplicity of the "original" protein, e.g. a di-, tri-, or tetramer of said protein into a suitable host, and subsequent expression of this low allergenic polymeric form of the protein. Alternatively a DNA sequence can comprise a multitude of codings for different enzymes, arranged in one combined sequence giving one combined protein. This DNA sequence is inserted into a suitable host, with subsequent expression of this low allergenic polymeric form of the combined protein.

The invention furthermore relates to the production and commercialization of such modified proteins. Of special interest is here industrial enzymes, food/feed proteins, proteins for use in householding and medicine.

A third aspect of the invention relates to compositions comprising the low allergenic proteins of the invention. Such as food/feed compositions, detergent compositions, or compositions used in therapy and/or diagnosis in the human or animal body.

A fourth aspect of the invention relates to the use of above mentioned compositions.

BRIEF DESCRIPTION OF THE DRAWING

The invention is described in more detail in the following parts of the specification with reference to the Examples and the Figures, where

Figures 1 to 5, show proteins according to the invention where the size of protein is increased through the methods of the invention.

In Fig.1. two identical proteins are being reversibly bound through a "linker" protein or peptide, Whereby the total size of the combined molecule exceeds the limit for penetration of the membrane.

Fig.2. shows the reversible linkage of three identical proteins.

Fig.3. shows the irreversible linkage of three identical proteins.

Fig.4. shows a number of monomers, identical to the original

molecule, linked together via a spacer consisting of a number of amino acids.
The linking amino acids will be breakable or degradable, f.ex. using enzymatic digestion specifically reacting with this sequence of amino acids.

Fig.5. shows a structure where the protein and the linking molecule are co-produced and react with each other to form larger molecular units.

DETAILED DESCRIPTION OF THE INVENTION

The invention relates generally to the allergic potential of proteins.

Proteins introduced to the wet epithelia will if they penetrate the membrane barrier pose the risk of stimulating the immune system. The stimulation of immunocompetent cells in these regions will often lead to production of IgE in humans, and antibodies with comparable action in animals. If IgE is being produced there is a risk of developing symptoms of allergy. On the other hand, the same molecules introduced subcutaneously or intraperitoneal will normally stimulate production of IgM and IgG. Therefore the way of presentation of the protein is of importance for the risk of developing symptoms of allergy.

Essential criteria for a protein stimulating the immune system are:

A) it is presented to the immunocompetent cells in the body,
B) it is foreign to the body,
C) it is of a size that allows cell-cell cooperation in the immune system leading to a reaction.

All three criteria will in many cases be fulfilled with proteins being produced by conventional present day production

methods.

For many industrially produced proteins the criteria (A) and (B) cannot be eliminated and the present invention therefore focus on the size of the molecule. The presentation of f.ex. dust form protein will be through the wet epithelia of especially the nose, but also pharynx, larynx, lungs and the gastro-intestinal system.

According to the invention protein molecules are increased in size so as to disable the penetration of the membrane barrier of the wet epithelia.

The size increase can basicly be obtained in five different ways:

1) By combining the protein with a reversibly binding protein or peptide, e.g. an enzyme inhibitor or an antibody or parts hereof, that together with the protein has a size too large to penetrate the membrane barrier. This can be achieved either by simple combination of one protein molecule with a protein/peptide binding molecule, or using protein/peptide binding molecules with 2 or more binding sites for protein molecules. In either way the total size will have to exceed the size for penetration in order to qualify for being a low allergenic protein of the invention. In later use of the protein the protein/peptide binding molecule is split off. The object protein persists or resumes its original activity/functionality. See Fig. 1 where two identical object proteins are being reversibly bound by another "binding" protein or peptide. Hereby the total size of the combined molecule is exceeding the limit for penetration of the membrane. Any other combination of object protein and binding protein/peptide can be used. The important parameter, besides the size, is the reversibility of the binding and the persistent or resumed activity/-functionality of the object protein.

2) By binding a reversible di- oligo- or multimerization linker molecule to the protein molecules. The proteins will therefore still be functioning as proteins with or without the linker split off, but their size will exceed the penetration possible size. See Fig. 2 where a reversible linkage of three identical object proteins has been made. The size of the linker can or cannot be of importance. There can be any number from di- to multimerization of proteins to the linker. The important parameter again, besides the size, is the reversibility of the linking and the persistent or resumed activity/functionality of the object protein.

3) By binding a small molecule/ligand to the protein, this molecule/ligand being recognizable by a di- oligo or multimerization linker molecule. The linking molecule will bind such molecule/ligand and hereby link two or more molecules together reversible or irreversible.

4) By binding a non-reversible di- oligo- or multimerization linker molecule to the protein molecules. The proteins will still be functioning in the linked conformation, but will be unable to penetrate the wet epitheliae. See Fig. 3, where an irreversible linkage of three identical object proteins has been made. The size of the linker can or cannot be of importance. There can again be any number of proteins on the linker from di- to multimers. The important parameter, besides the size, is the irreversible linking and the persistent activity/functionality of the linked object proteins. The oligomerization can be by chemical, biochemical or enzymatic means.

5) By changing the genomic material to include more than one coding DNA sequence for the protein/peptide. This will preferably be within the one and same initiation and termination regulatory signals in the replicable entity. In between the single coding regions for the molecule can

be non-informative basepairs, basepairs coding for extra amino acids, or the coding regions can be consecutive.

The optional extra amino acids may act as a linker or spacer between two copies of the original molecule. The extra amino acids will preferably not interfere with the 3-dimensional structure or the activity of the original molecule incorporated. Flexible, uncharged amino acids like Glycine, Alanine or Serine are preferred. The multiple copies are therefore at the mRNA or at the protein level joined to make one molecule with increased size.

The larger molecule can or cannot be breakable into smaller subunits, corresponding to the molecule of origin. This will enable the same or nearly same activity as the molecule of origin. In Fig. 4 the eventual molecule corresponds to a number of monomers, identical to the original molecule, linked together via a spacer consisting of a number of amino acids. These new linking amino acids are preferably small uncharged amino acids, and will give small or no change the activity and the 3 dimensional structure of the monomers. The change is in the genome, and hereby the number of monomers in the new molecule is determined strictly to the desired number.

The linking amino acids will be breakable or degradable, f.ex. using enzymatic digestion specifically reacting with this sequence of amino acids.

The activity of the new oligo- or multimere is identical or comparable to the original monomer. After a possible splitting of the oligo- or multimere, to become monomer with or without "tails" of degraded linker amino acids, the activity is identical or comparable to the original monomer.

6) By including one or more extra molecules in the replicable

organs of the host organism in which the object molecule is produced. This change of host will eventually mean production of the object molecule together with another molecule, that preferably will bind to the object molecule, reversibly or irreversibly. Therefore the product both before and after purification will be larger than the original molecule. In Fig. 5 the organism producing the original molecule is changed to become producer of another molecule too. The change therefore is in the genome of the organism. The co-produced molecules react with each other and form larger molecule units. Any numbers can be used in combination.

The important parameter besides the molecular size, is the reversibility of the bonding, and the sustained or resumed activity of the original molecule linked or unlinked to the added molecule.

7) The size increase can include any of the above mentioned methods (1) to (5) alone or in combination, and can involve more than one type of protein being bound/linked, f.ex. two different enzyme molecules being linked to one linker.

The methods for binding proteins in (1) can for example be using antibodies of parts hereof, or using biotinylation of the protein and bridgeformation with the quadrovalent avidin or streptavidin, or any other conventional reversible binding, or in the case of enzymes an inhibitor to the enzyme(s).

The methods for linking proteins in (2) and (3) can in example be using methods like the ones described in :"Ultrogel, Magnogel and Trisacryl. Practical guide for use in affinity chromatography and related techniques. (1983) LKB+IBF. Reactifs IBF- Societé Chimique Pointet-Girard. 35, avenue Jean-Jaurès, 92390 VILLENEUVE-LA-GARENNE, FRANCE.

The reversible bonds and links will be breakable through one of

the following procedures:

a) change of pH,
b) change of temperature,
c) change of ionic strength,
d) change of molarity, e/di-lution,
f) addition of competitor for binder/linker,
g) degradation of binder/linker molecule, or
h) any combination of these.

Test of sustained activity of proteins or peptides

The molecules from any of the procedures (1) through (6) above is tested for activity.

In the procedures giving irreversible binding of the original molecule, or where multiple copies of the molecule is represented in the genome giving molecule oligo- or multimeres, the activity must be sustained directly as such in the larger molecule.

In the procedures giving reversible or breakable binding or linking of original molecules, the activity of the molecules must be a/sustained in the larger molecule, or, b/resumed after splitting the larger molecule into molecules identical or comparable to the molecule of origin.

In the above mentioned procedures (3), (4) and (6) the activity is measured with the larger molecule, whereas molecules from procedures (1), (2), (5) and (6) the activity is measured with both the larger molecules, and with the monomers after reversing or breaking the bonds/links.

Test of reduced immunologic/allergenic potential

The molecules are after change tested for their immunologic/allergenic potential, and selected according to reduction hereof. The new molecule(s), together with molecule of origin and control material is presented to test animals through one or more of the following ways : a/intra dermal b/sub cutaneous,

c/intra venous, d/intra nasal(inhalation), e/per oral, f/intra tracheal or g/intra peritoneal.

The allergenic potential will be measurable with preferably d/ and f/ or partwise e/, whereas the immunologic potential will be measurable in all techniques.
For means of dosing the test material, please refer to standard protocols and for f/ to examples later in this text.

The response of the animals is measured, and the responses compared to evaluate the effect of change of molecule size. The responses can be measured as : differential count of blood/-lymphoid cells, lymphocyte stimulatory index, specific antibodies in animal sera (the quality and quantity), or any other means of measuring the immunologic status of the stimulated animal.

The molecules are bound either to another protein or peptide or to each other through a linker. After this bonding or linking the new molecule will have a size, that impedes or stops any penetration over the membrane barrier in the wet epithelia.

The molecules are monitored for immunologic stimulatory potentials with and without the change on the wet epithelia and as control by injection. The optimal change(s) is selected for use and implemented in either production or processing of the proteins. The final product is again tested for immunologic potential.

Methods for monitoring and testing the potentials are immunological and proteochemical, both in vivo and in vitro methods can be used.

The changes selected for size increase of proteins, can be implemented either in the production step of the proteins, or in any later processing step of these proteins. It can therefore be implemented on the genomic level of the production

organism, included in the food/medium for growing these organisms, or used to change proteins post-production, before or after a possible purification of the protein(s).

EXAMPLES

EXAMPLE 1

In this example the irreversible coupling of proteins to each other is discussed. The coupling will preferably not change the molecular activity.

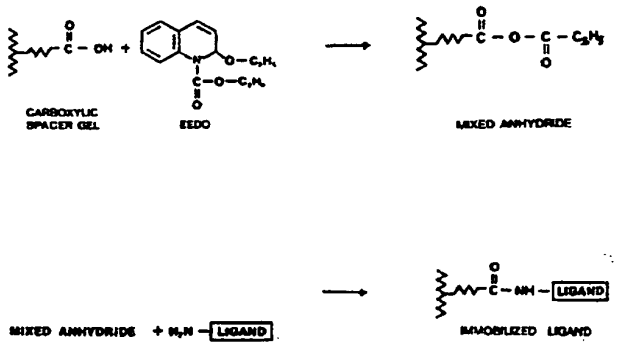
The means of coupling is through the use of bivalent linker molecules i.e. homo- or heterobifunctional reagents. For exhaustive information on bifunctional reagents see among others ref.s 1 & 2.

Any one of these coupling methods can be used. The selection of which one to choose depends on the chemical composition and stability of the molecule to be coupled.

Method

Glutaric acid is reacted with EEDQ (= N-ethoxycarbonyl-2-ethoxy-1,2,dihydroquinoline) (ref 1). A mixed anhydride is formed, that in its turn will react with a primary amino group in a peptide or protein. Through a condensation reaction the glutaric acid is coupled to the peptide or protein.
Hereby glutaric acid acts as a bridging molecule linking two f.ex. peptides.

The reactions are illustrated in the following figs, taken from ref 1 (where the discussion in on protein binding gels).



1. In the first step glutaric acid is reacted with EEDQ in excess in ethanol. After one hour of reaction formic or acetic acid is added (equimolar to EEDQ) to stop the reaction. The mixed anhydride is now formed, and further reaction with excess EEDQ is stopped.

2. The peptide or protein is dissolved in a waterphase or mixed water/ethanol solution. The peptide or protein is added to the mixed anhydride. The peptide or protein must be in molar excess compared to the initial glutaric acid.

   The reaction is incubated at least one hour, preferably over night with or without agitation.

   In this reaction f.ex. two proteins are being linked together via glutaric acid.

3. The product of step 2 is purified using size or other chromatography. The correct fraction is selected according to molecular size.

   If necessary the procedure is run over with the purified product of step 3 as the ligand, in replace of peptide or

protein as listed there.

In one or more runs of the procedure, the molecular weight is increased to exceed the limit for being penetrable over the wet epithelias.

EXAMPLE 2

The theory behind this is identical to example 1.

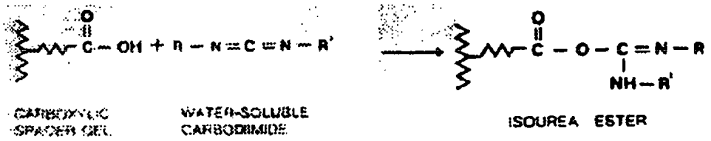
Method

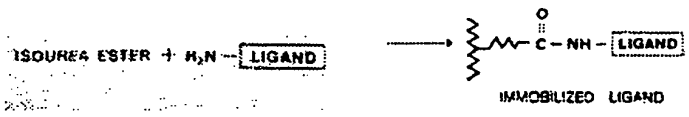
A dicarboxylic acid like glutaric, ketoglutaric, sebacic or like acid is used for linking peptides or proteins. In the following sebacic acid is be used.

A carbodiimide is reacted with sebacic acid, whereupon an isourea ester is formed. Residual carbodiimide is exhausted with a monocarboxylic acid like formic or acetic acid. The peptide or protein is introduced, and through a condensation reaction this is bound to the former sebacic acid in a chemical bond.

Hereby sebacic acid is linking two f.ex. proteins together chemically.

The reactions are illustrated in the following figs (see ref 1) :





1. Sebacic acid is diluted in water to the desired molarity, pH is adjusted to slightly acidic. EDCI (= 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride, or, CMCI (= 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide-metro-p-toluene-sulphonate is added, either in excess. The isourea ester is now formed.

   Leave to react for one hour. Formic or acetic acid is added to exhaust residual carbodiimide.

2. The peptide or protein to be coupled is added. This reacts with the isourea ester through a condensation reaction where peptide or protein is chemically bound to the former sebacic acid. As sebacic acid is bifunctional it will link to two peptides or proteins acting as a bridge between these.

3. The product of step 2 is purified using size or other chromatography. The correct fraction according to molecular size is selected.

   If necessary the procedure is run over, with the purified product of step 3 as the ligand, as replace for peptide or protein mentioned there.

   In one or more runs of the procedure, the molecular weight is increased to exceed the limit for being penetrable over the wet epithelias.

EXAMPLE 3

In this example the irreversible linking of another, small molecule to be object molecule is used as a preactivation step. Hereafter the preactivated molecule is linked using a di-oligo- or multimerization linker molecule that binds reversible or irreversible.

The activity of the object molecule is retained during the manipulation or, resumed after monomerization of the object molecule by splitting off the linker.

Method

The object molecule is being biotin labelled using Biotin N-HydroxySuccinimide (BNHS) reagent. Hereby the molecule is added the small biotin molecule (mw 241 D) in low incorporation quantity, preferably ratio 1 biotin to 1 object molecule.

The residual unused BNHS reagent is extinguished using an amino containing molecule like Glycin, and the free biotin molecules are removed.

Next step is addition of the linking Streptavidin or Avidin, that will be able to bind the biotin labelled object molecule in reversible bonds, although with very high binding strength.

1. The object molecule containing free amino groups is reacted with BNHS reagent at neutral to alcaline pH. The molecular ratios must be more than 1:1, preferably more than 1:15 during the reaction, to give an incorporation of 1:1 eventually.

2. A solution of Glycin is added to the reaction mixture of point 1 above. The molecular ratios BNHS to Glycin must exceed 1:1, preferably 1:8.

3. Dialyse or gelfiler the product of point 2 above to remove

free uncoupled Biotin from the reaction mixture.

4. Aliqout the reaction mixture of point 3 above, add to a few of these linking Strptavidin or Avidin giving mixing ratios of linker to object molecule of 1:100, 1:40, etc. Find the best ratio using sized exclusion chromatography or electrophoresis or other mw determining procedure. Use mixing ratios will possibly give linked products of ratios linker to object molecule of 1:1, 1:2, 1:3 and 1:4, wherefore a spectrum of molecular sizes can be obtained.

5. Purify the eventual product of point 4 above by e.g. size exclusion chromatography, verify the mw by e.g. electrophoresis.

6. The correct size is selected according to molecular size.

Further examples, using chemical coupling

Homo- or heterobifunctional reagents is used together with f.ex. dicarboxylic- or diamino containing compounds acting as linkers.

The bifunctional reagents will be any one listed in the references or any other.

The linkers will be any compound capable of being reacted upon by the bifunctional reagents. Examples are putrescine, spermidine or spermidine (all diamino compounds), or, glutaric, ketoglutaric (alpha/beta) or spermine (all dicarboxylic acids), or any other compound with thiol- hydroxyl- carboxyl- or amino groups combined in one linker, or with two identical groups in the same linker.

Examples using breakable linkages

Any one of the above listed methods, using a linker with scissile bond, or, a linker that is breakable. All linkages will preferably not change the activity of the peptide or

protein.

A scissile bond is broken by changing the dilution(i.e. the molarity), temperature, ionic strength, pH or other.

A breakable linker is a linker that itself breaks apart by changing the temperature, ionic strength, pH or other.

The break can also be induced by enzymes capable of digesting or splitting the linker, preferably without altering the peptide or protein coupled to the linker.

Furthermore the linkage can be obtained using another peptide/-glycopeptide or protein/glycoprotein. This molecule will be active like f.ex. an antibody or an enzyme inhibitor, preferably without having any immunological potential of its own.

The bond to the active molecule can be broken by any of the above mentioned means.

REFERENCES

1. Ultrogel, Magnogel and Trisacryl. Practical guide for use in affinity chromatography and related techniques. 2nd Edition. Réactifs IBF - Sociéte Chimique Pointet-Girard. 35, Avenue Jean-Jaurés. 92390 Villeneuve-La-Garenne. France (book).
2. Tae,H.Ji (1983) Bifunctional Reagents. Methods in Enzymology. Vol. 91. p.580-605 (article).
3. Hudson, L. and Hay,F.C. (1991). Third edition. Practical Immunology. Blackwell Scientific Publications (book).
4. Henderson,R.F.(1988). Use of Bronchoalveolar Lavage to Detect Lung Damage. in : Toxicology of the Lungs. Eds. Gardner, D.E.; Crapo,J.D. and Massaro, E.J.. Raven Press N.Y. (book).

PATENT CLAIMS

1. A low allergenic protein comprising an oligomeric form of the parent monomeric protein.

2. The protein of claim 1, wherein the size of said oligomer is above 30 kD.

3. The protein of claim 1, wherein the size of said oligomer is above 40 kD.

4. The protein of claim 1, wherein the size of said oligomer is above 50 kD.

5. The protein of claim 1, wherein the size of said oligomer is above 60 kD.

6. The protein of claim 1, wherein the size of said oligomer is above 70 kD.

7. The protein of claim 1, wherein the size of said oligomer is above 80 kD.

8. The protein of claim 1, wherein the size of said oligomer is above 90 kD.

9. The protein of claim 1, wherein the size of said oligomer is above 100 kD.

10. The protein of any of the claims 1 to 9, wherein said oligomer has been formed by linking said monomeric parent protein molecules to each other.

11. The protein of claim 10, wherein said linking has been formed by the use of a linker or spacer molecule

12. The protein of claim 10, wherein said linking has been

formed directly.

13. The protein of claim 10, wherein said linking has been formed through peptide bonds between the C-terminal of a first monomer and the N-terminal of a second monomer, and optionally introducing one or more amino acid residues between the monomers.

14. The protein of claim 13, wherein said peptide bond and optional amino acid residues has been established by the monomers being co-expressed as one single protein.

15. The protein of any of the previous claims, wherein said oligomer has substantially retained its activity, or is capable of having its activity reestablished.

16. The protein of any of the previous claims, wherein said link or bond is reversible.

17. The protein of any of the previous claims, wherein said link or bond is irreversible.

18. The protein of any of the preceding claims, wherein said protein is an enzyme or a hormone.

19. The protein of claim 18, being an enzyme.

20. The enzyme of claim 19, being a medical enzyme.

21. The enzyme of claim 20, being selected from the group comprising Factor VII, Factor VIII, Factor IX, Protein C, Thrombomodulin, Thrombin, or active fragments thereof.

22. The enzyme of claim 19, being an industrial enzyme.

23. The enzyme of claim 22, being a hydrolase.

24. The enzyme of claim 22, being selected from the group comprising proteases (metallo, acid, neutral or alkaline), lipases, cellulases, amylases, lyases, xylanases, pectinases, polygalacturonases, oxidases, lacases, oxidoreductases, or peroxidases.

25. A process for the production of a low allergenic protein according to any of the preceding claims, comprising the steps of:

i) bringing the monomeric parent protein molecules together to form an oligomer,

ii) testing said oligomer for its ability to perform the desired activity, and

iii) testing said oligomer for its allergenicity.

26. A protein variant produced by any of method of claim 25.

27. A composition comprising a low or non-allergenic protein according to any of the claims 1 to 24 or 26.

28. The composition of claim 27, where the composition is for industrial use.

29. The composition of claim 27, where the composition is for householding use.

30. The composition of claim 27, where the composition is for food/feed use.

31. The composition of claim 27, where the composition is for medicinal use.

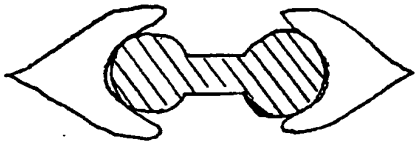


Fig. 1

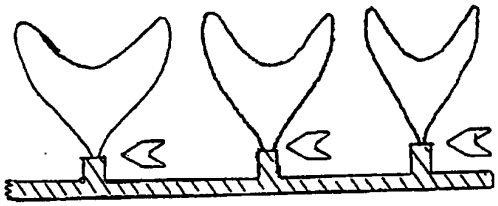


Fig. 2

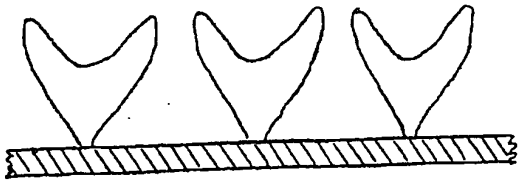


Fig. 3

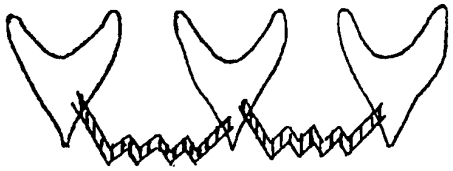


Fig. 4

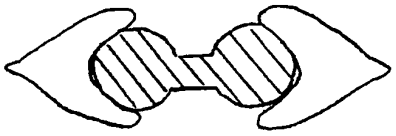


Fig. 5

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00344

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: C07K 3/06, C12N 9/96
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: C07K, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, BIOSIS, DBA, WPI, CLAIMS, US PATENT FULLTEXT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EP, A2, 0215662 (MAEDA, HIROSHI), 25 March 1987 (25.03.87), page 5, line 7 - page 6, line 13; page 29, line 8 - line 13; page 36, line 16 - line 25	1-31

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
3 February 1994	04 -02- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Jonny Brun Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 93/00344

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

The wording "A low allergenic protein" is to broadly formulated to permit a meaningful search. The search on claims 1-17 has therefore been incomplete (See Art. 6).

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

30/12/93

International application No.
PCT/DK 93/00344

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A2- 0215662	25/03/87	JP-A- 63041426	22/02/88
		US-A- 4844897	04/07/89
		JP-A- 62061926	18/03/87

Form PCT/ISA/210 (patent family annex) (July 1992)